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13. ABSTRACT ***(Maximum 200 Words)***

Behavioral, neurochemical, and immunocytochemical studies characterized the possible role of insecticide exposure in the etiology of Parkinson's disease as it may relate to Gulf War Syndrome. Chlorpyrifos (CP) and permethrin (PM) were given 3 times over a two week period by injection (CP subcutaneous and PM intraperitoneal) with or without a single dose of the Parkinsonian neurotoxin, MPTP (10-30 mg/kg, intraperitoneal). Some synergism was observed at 30, but not 10 mg/kg MPTP with respect to behavior and dopamine depletion. PM (0.8-1.5 mg/kg) increased dopamine transporter protein expression 30%, similar to its ability to increase dopamine uptake and GBR12935 binding at these doses. Time course studies showed that the magnitude of transporter expression increased to roughly double the value of controls 4 weeks after treatment, suggesting a slow process underlies this effect. In immunocytochemical studies, PM at 200 mg/kg, and mixed doses of PM and CPF (200 and 50 mg/kg, respectively) showed no change in tyrosine hydroxylase staining, but did cause an increase in glial fibrillary acidic protein consistent with neuronal injury. Similarly, PM (200 mg/kg) and CPF (75 mg/kg) down regulated both high and low affinity binding sites for the nicotinic receptor ligand, epibatidine. Further work is underway to characterize this down regulation.

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INTRODUCTION

This study is focused on the neurotoxic actions of the insecticides permethrin (PM) and chlorpyrifos (CPF) as they relate to the development of Parkinson's Disease (PD). These compounds possess properties that could damage the nigro-striatal system, which is the primary brain lesion in PD (Bowman and Rand, 1980). The research is assessing the ability of each compound alone, or in combination, to directly induce neurochemical or neuropathological hallmarks of PD. In addition, since PD is hypothesized to have a multifactorial etiology (Butterfield *et al.*, 1993), these compounds are also being tested for their ability to synergize the actions of the established Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This approach will determine any ability of the insecticides to accelerate or intensify idiopathic disease processes. Experiments are performed on the C57BL6 black mouse, which when given MPTP is a valid rodent model for the development of PD (Heikkila and Sonsalla, 1992). For each treatment group, effects consistent with metabolic insult and changes in cholinergic and dopaminergic neurotoxicity in the striatum are measured. Cell stress in striatal nerve terminals is evaluated by measurements of mitochondrial function. Other neurochemical studies measure effects specific to the dopaminergic pathways in the striatum, including the amounts of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid, as well as the ability of isolated nerve terminals to transport dopamine. Because of the anticholinergic effect of chlorpyrifos, we also measure acetylcholinesterase activity, and the density/function of muscarinic and nicotinic receptors following insecticide treatment. Neuropathology studies are focused on any gross changes in immunocytochemical markers for glial fibrillary acid protein (GFAP). Other antibody labeling studies will assess effects specific to the dopaminergic system, including antibody labeling of tyrosine hydroxylase and the dopamine transporter (DAT). These studies represent a unique combination of research approaches and will provide a comprehensive and integrated evaluation of the possible Parkinsonian or neurodegenerative effects of these insecticides.

BODY

The experiments for the third year were, in part, completion of studies related to Objective #1, which is to characterize any effects on biomarkers of PD over a range of doses of PM and CPF. Other studies have addressed objectives related to possible synergistic interactions of toxicants (Objective #2), as well as the reversibility of effects on various biomarkers (Objective #3). At the end of the last annual report, we mentioned that we had a number of frozen brain tissue samples from treated mice that would be used for additional radioligand binding studies, and this area is where we began work in year three. We have performed only a portion of the experiments originally planned for the third year. The turnover of postdoctoral scientists was the main factor limiting our ability to complete all of the scheduled experiments. The position vacated by Dr. Dan Karen (Oct., 2000) was offered to Dr. Jing Shen, a Chinese M.D., who is currently working at the Tokyo Women's Hospital in the pediatric cardiology unit. We spent several months trying to secure an appropriate visa for Dr. Shen, which I believe was complicated by the collision of Chinese and American military aircraft early last year. In April, we abandoned efforts to recruit Dr. Jing and offered a position to another Chinese scientist Jinghong Kou, who, after a 3 month delay was issued a visa and joined the project in late august of 2001. Because new people have joined the group every few months, we have had to spend an inordinate amount of time in training. This past year, we sought, and were granted, a one year, no-cost grant extension to finish the work. Results for year three of the project are organized by alphabetical listing of objectives, as given in the amended grant proposal and in the annual reports for previous years.

Treatment of mice in year three for studies of insecticides alone, and insecticides with MPTP, was performed as described in the proposal, and is illustrated in Figures 1 and 2, respectively. Treatment groups for the procedure given in Figure 1 were controls, PM, CPF, or PM+CPF, but these studies were limited in number. Because PM and CPF did not deplete dopamine when given alone, we emphasized the MPTP combination studies. Treatments for the procedure given in Figure 2 were controls, MPTP, MPTP+PM, MPTP+CPF, or MPTP+PM+CPF.

One of the observations we reported last year was greater than expected mortality in treatments with MPTP at 30 mg/kg, which suggested that lower doses needed to be used. Also, the reviewer of last year's report suggested that we use a range of doses of MPTP in the synergism experiments. Such studies were already underway when last year's report was filed, and we have added synergism studies at 20 and 10 mg/kg MPTP. No lethality occurred at the lower doses of MPTP used in the past year.

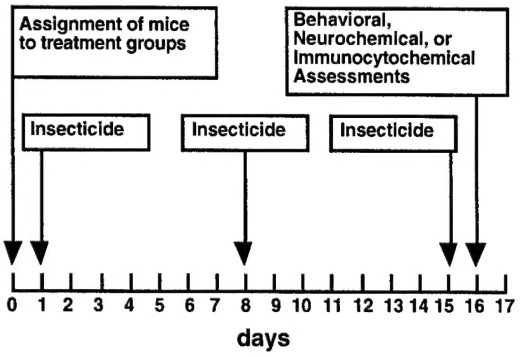


Figure 1. Treatment regime for studies with insecticides alone (PM, CPF) or in combination (PM+CPF).

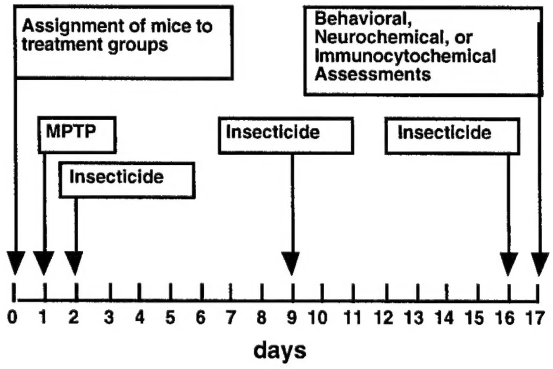


Figure 2. Treatment regime for studies of insecticides in combination with MPTP (MPTP, MPTP +PM, MPTP + CPF, MPTP+PM+CPF). Animals receiving MPTP alone were also given insecticide vehicle.

a. Assess toxicant effects on dopamine titers and turnover by measuring the dopamine and 3,4-dihydroxyphenyl acetic acid (DOPAC) content of the striata from treated mice.

Methods: Dopamine and DOPAC content of the striatum were determined by HPLC with electrochemical detection (Hall *et al.*, 1992). In all studies, mice were sacrificed by cervical dislocation, and the striata rapidly dissected and weighed. The striata are homogenized in 5% trichloroacetic acid (TCA) with 62.4 ng of 3,4-dihydroxybenzylamine (DHBA) as the internal standard, and stored at -70 °C until analyzed. The samples were thawed, centrifuged to pellet the membranes and the supernatant analyzed for dopamine and DOPAC by HPLC through a C_{18} column. The mobile phase consisted of [90% aqueous (0.1 M sodium acetate, 2 mM heptanesulfonic acid, 0.33 mM EDTA, adjusted to pH 4 with glacial acetic acid)/10% methanol (v/v)], at a flow rate of 1 ml/min. Quantitation was determined from standard curves of peak height ratios (dopamine/DHBA) obtained from stock solutions containing dopamine at multiple levels and DHBA as the internal standard. Data were analyzed by analysis of variance (ANOVA) with Student-Newman-Keuls means separation test.

Results and Discussion: New studies on dopamine depletion with insecticides at lower doses of MPTP are shown in Figures 3-5. In these experiments, 20 mg/kg MPTP showed significant depletion of striatal dopamine (>70%), that was not enhanced by the insecticides PM and CPF at a range of doses (Fig. 3). In a few cases, we actually observed a small increase in striatal dopamine after insecticide treatment, but the effect was not consistent, nor dose-dependent. In addition, we observed no insecticide-dependent depletion of DOPAC in any of these studies (data not shown). In studies using 10 mg/kg MPTP, depletion of dopamine was about 37% (Fig. 4), and in this case also, there was no synergism by high doses of insecticides. We then combined treatments of a low dose of PM with MPTP (Fig. 5). This experiment was performed because we have consistently observed a PM-dependent increase in dopamine transport, and we assumed that greater transporter activity would increase the amount of MPP^+ taken up by the dopaminergic nerve terminals. This result, in turn, should cause a greater depletion of dopamine. In these studies, MPTP (30 mg/kg) depleted about 70% of the dopamine, and co-application with 1.5 mg/kg PM had no synergistic effect. This result is perhaps not too surprising, since we have observed a relatively modest 30% increase in dopamine transport in PM-treated mice at this dose. A related issue may be a concomitant toxicant-induced increase in VMAT2 expression, such as we have observed with the organochlorine insecticide heptachlor (Miller et al., 1999). Increased expression of this vesicular amine transporter, would negate a synergistic effect on DAT expression by increasing sequestration of MPP^+ by the synaptic vesicles, since MPP^+ is known to be a substrate for the VMAT2 (Yelin and Schuldiner, 1995). We have not yet run this experiment at a lower MPTP dose, and have new results that suggest that the timing of the treatments we used here may not be optimal for observing an effect of DAT expression on MPTP toxicity. This issue will be discussed later in the report.

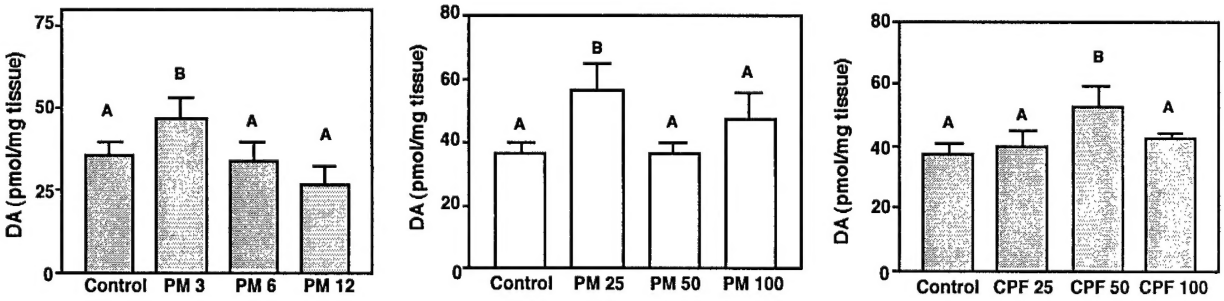


Figure 3. Dopamine titers in mouse striatum following co-treatment with toxicants. Control = 20 mg/kg MPTP, and other treatments included the indicated doses of PM or CPF given in combination (mg/kg). Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different.

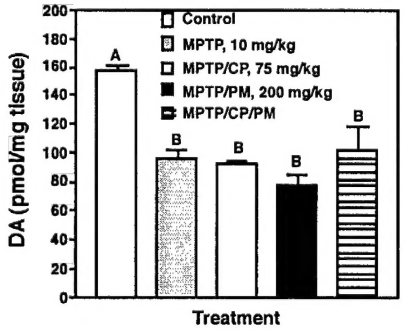


Figure 4. Dopamine titers in mouse striatum following injection with toxicants. Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different.

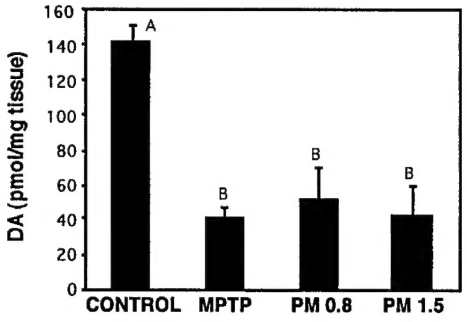


Figure 5. Dopamine titers in mouse striatum following co-treatment with toxicants. MPTP= 30 mg/kg , other treatments included the indicated doses of PM given in combination. Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different.

b. Assess effects on the density and kinetic properties of dopamine transporters in striatal synaptosomes from treated mice.

Methods: For both dopamine uptake and radioligand binding studies of the DAT, we prepared striatal synaptosomes *ex vivo* from treated mice using the methods of Bloomquist *et al.* (1994). For studies of dopamine uptake kinetics, synaptosomes were incubated with [^{3}H]dopamine (Amersham Corp.) for 2 min. Transport was terminated by dilution with 3 ml of ice cold buffer and immediate vacuum filtration on glass fiber filters. The filters were then washed with cold buffer and the amount of radioactivity on the filters determined by liquid scintillation spectrometry. Nonspecific uptake was determined in Na^+-free buffer following the method of Krueger (1990). For labeling of dopamine uptake sites, we used an equilibrium binding assay with the established uptake inhibitor [^{3}H]GBR12935 (NEN Research). Binding employed incubation to equilibrium, followed by filtration, rapid washing, and liquid scintillation counting. Nonspecific binding was estimated with saturating concentrations of the closely related analog GBR12909. Uptake and radioligand binding data were analyzed using InPlot™ (GraphPad Software, San Diego, CA).

Although our previous studies with GBR binding were consistent with uptake measurements, we have added Western blot analysis since it is related to the immunocytochemical objectives of the project, and it provides savings in the number of animals required. Procedures are as follows. Murine striatal synaptosomal proteins (10 μg protein per lane) were separated on a 10 % SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was subsequently incubated in 4% dry milk, followed by overnight incubation in a rat monoclonal primary antibody (Chemicon) at 4° C. Primary antibody was diluted to 1:1000. The blots were then incubated in a peroxidase-linked secondary antibody (anti-rat; Sigma Chemical) for one hour at room temp. Blots were developed using the chemiluminescence detection method (Amersham Pharmacia), and exposed to photographic film for varying lengths of time from 5-20 sec. Blots were originally incubated in primary antibody for 1 hour at room temp. Later blots were incubated overnight at 5° C, with no differences in the outcome of the blot. Typically, at room temperature, all binding should occur in the first hour of incubation. Longer incubation times do not significantly change the intensity of staining. After transfer of proteins to nitrocellulose, the blots are stained with Ponceau S dye to ensure transfer of the proteins to the membrane. This serves an added purpose of ensuring that equal amounts of protein have been loaded onto each lane.

Results and Discussion: The blots obtained in our laboratory show two closely spaced bands when blotted with a rat monoclonal antibody (Fig. 6). While only one DAT band has been reported previously (Miller *et al.*, 1999), our observations may be due to the presence of more than one DAT

previously (Miller *et al.*, 1999), our observations may be due to the presence of more than one DAT glycoprotein in the mouse, or an artifact of membrane preparation. We heated the membranes to ensure denaturation (95 °C, 4 min), which is standard Western blot methodology (Harlow and Lane, 1999). Since the multiple banding is reduced without heating (Fig. 6), we will run our blots without this preparatory step and with a proper molecular weight marker to make certain that our DAT protein is indeed in the 80 kDa range.

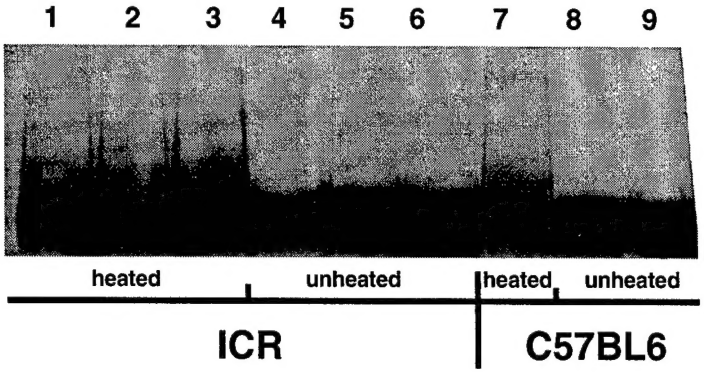


Figure 6. Representative Western blot of striatal protein from ICR (lanes1-6) and C57BL6 (lanes 7-9) mice labeled with DAT antibody. Heating the samples may be related to the multiple bands observed.

Densitometry scans of Western blots (Fig. 7) showed an increase in DAT protein that correlated with the increase in dopamine uptake observed previously at 1.5 mg/kg PM (mean of four experiments: 33% above control). Of course, the optimal dose for observing increased uptake varies a bit between different cohorts of mice, and represents experimental variability. In blots of synaptosomal protein from mice given 0.8 mg/kg PM, both bands 1 and 2 were increased 15% each, for a total of 30%. At 1.5 mg/kg, bands 1 and 2 were increased 21 and 8%, respectively. Thus, in each case there was good agreement between DAT protein and transport.

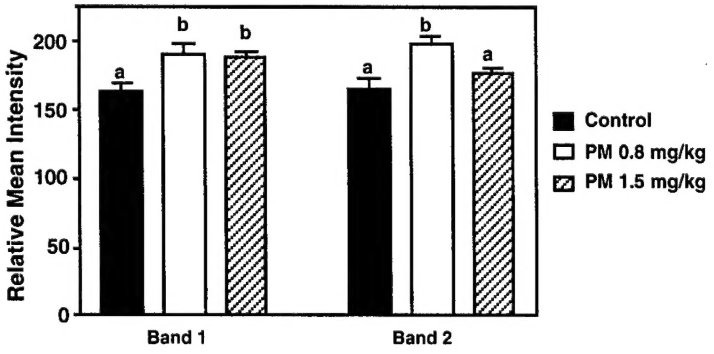
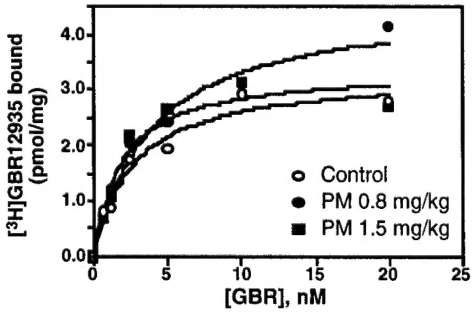


Figure 7. Changes in antibody staining intensity following treatment with PM. Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different.



	Control	PM 0.8 mg/kg	PM 1.5 mg/kg
Variables			
BMAX	3.308	4.608	3.363
KD	2.652	3.867	1.815
Std. Error			
BMAX	0.2809	0.3916	0.3045
KD	0.7082	0.9234	0.5785
95% Confidence Intervals			
BMAX	2.585 to 4.030	3.601 to 5.615	2.580 to 4.146
KD	0.8308 to 4.473	1.493 to 6.241	0.3275 to 3.302
Goodness of Fit			
Degrees of Freedom	5	5	5
R squared	0.9638	0.9734	0.9491
Absolute Sum of Squares	0.2533	0.3166	0.4271
Sy.x	0.2251	0.2516	0.2923

Figure 8. GBR12935 binding to *ex vivo* striatal synaptosomes prepared from PM-treated mice. Left, isotherm binding plots; Right, kinetic and statistical parameters for the data.

Changes in DAT protein in Western blots were reasonably correlated with the maximal binding of [^{3}H]GBR12935, a ligand that labels the DAT (Horn, 1990). In these studies (Fig. 8), B_{max} was increased 39% at 0.8 mg/kg PM, which was significantly different from controls (T-test). Binding was increased only about 2% at 1.5 mg/kg PM. There was no significant effect on K_d in either case. In the previous report, we calculated a B_{max} of 80 pmol/mg protein for [^{3}H]GBR12935 binding, due to a calculation error. The correct value given in Fig. 8 is similar to that reported by Horn (1990), who listed a K_d of 0.82 nM and a B_{max} of 5.5 pmol/mg protein for GBR12935 binding in rat striatum.

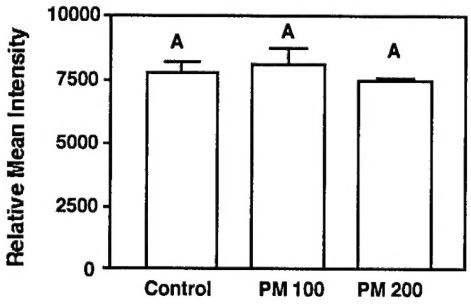


Figure 9. Lack of change in DAT antibody staining intensity following treatment with high doses of PM. Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different.

Experiments with [^{3}H]GBR12935 in PM-treated C57 mice (100 and 200 mg/kg) showed no difference from controls (Fig. 9, 2000 report), even though dopamine uptake was reduced at these doses. We have confirmed that there is no change in DAT protein levels in treated mice, as shown in Figure 9 above of the 2001 report. This finding is also in accord with DAT immunocytochemical labeling reported in the first year's report, which was not changed by treatment with 200 mg/kg PM. Taken together, these observations suggest that other mechanisms besides a change in density of the DAT is responsible for the reduced uptake. The synaptosomes may be able to transport the dopamine normally, but are unable to sequester it due to enhanced leakage. Pyrethroids are known to stimulate secretion of transmitters (Kirby *et al.*, 1999), but in resting synaptosomes this effect usually requires some stimulus (*e.g.*, veratridine activation of sodium channels). In addition, there may also be a PM-induced effect on cytotoxicity or respiration, since reduced complex I activity (MTT reduction assay) was observed previously (*Neurotoxicology* manuscript in press, see appendix).

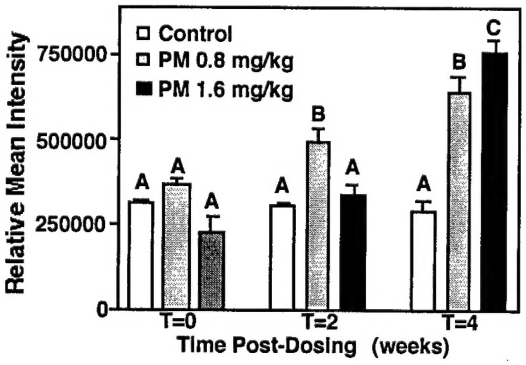
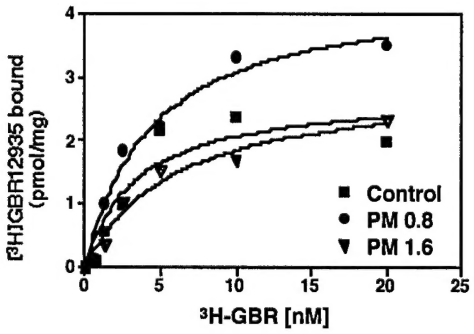


Figure 10. Time course in DAT antibody staining intensity following treatment with low doses of PM. Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different.

The extent of reversibility of the increase in DAT labeling was investigated (Objective #3) by analyzing DAT antibody labeling in tissues from mice sacrificed 24 hr, 2 weeks, and 4 weeks after the last treatment (Fig. 10). These are recent experiments, and were done on 11 month old mice, somewhat older than usual (7-9 months). Interestingly, we see a hint of an increase in DAT at 0.8 mg/kg PM 24 hr after treatment, but much greater increases after 2 weeks, and a doubling of DAT protein after 4 weeks. This slow time course is quite unexpected, and suggests that a slow time course for other biomarker changes may be occurring, and may have been underestimated by evaluating them 24 hr after treatment. A recent study by Lee *et al.* (2001) observed an interaction

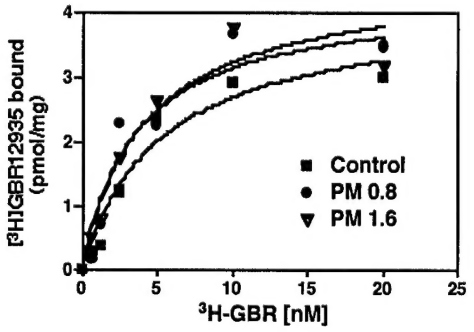
between the DAT and α-synuclein in cultured neurons. This interaction led to increased dopamine uptake and dopamine-induced cellular apoptosis, and would provide a mechanism whereby increased DAT expression by insecticides could play a role in the development of PD. It will be important to confirm that this upregulation in protein is mirrored by increased transport of dopamine, in order to establish that the protein is functional. The time course of this effect is also significant because PM should be cleared rapidly within a few days following treatment (Anadon *et al.*, 1991). This fact suggests that a process is set in motion by PM exposure that continues after clearance of the compound, resulting in a persistent upregulation of the DAT.

Changes in DAT protein levels in reversibility studies were paralleled by changes in the B_{max} for [^{3}H]GBR12935 binding at 2 weeks (Fig. 11), but not at 4 weeks (Fig. 12) for mice treated with PM. At 2 weeks posttreatment, B_{max} was significantly increased in the 0.8 mg/kg group, but not in the 1.5 mg/kg group. Although binding was numerically increased in both treatment groups at 4 weeks, the increase was not statistically significant. The greater variability in this experiment appears to be responsible (note the relative size of the Std. Error values in Figs. 11 and 12). No significant changes in K_d values were noted in any of the experiments.



	Control	PM 0.8 mg/kg	PM 1.6 mg/kg
Variables			
BMAX	2.763	4.399	2.947
KD	3.382	4.330	5.957
Std. Error			
BMAX	0.5358	0.4356	0.3697
KD	1.919	1.164	1.836
95% Confidence Intervals			
BMAX	1.385 to 4.140	3.279 to 5.519	1.997 to 3.898
KD	-1.551 to 8.314	1.337 to 7.322	1.237 to 10.68
Goodness of Fit			
Degrees of Freedom	5	5	5
R squared	0.8830	0.9717	0.9680
Absolute Sum of Squares	0.7007	0.3370	0.1511

Figure 11. GBR12935 binding to *ex vivo* striatal synaptosomes prepared 2 weeks after mice were treated with PM. Left, isotherm binding plots; Right, kinetic and statistical parameters for the data.



	Control	PM 0.8 mg/kg	PM 1.6 mg/kg
Variables			
BMAX	4.095	4.547	4.267
KD	5.155	4.038	3.547
Std. Error			
BMAX	0.5821	0.7096	0.5655
KD	1.884	1.747	1.356
95% Confidence Intervals			
BMAX	2.598 to 5.591	2.723 to 6.371	2.814 to 5.721
KD	0.3101 to 10.00	-0.4549 to 8.530	0.06204 to 7.033
Goodness of Fit			
Degrees of Freedom	5	5	5
R squared	0.9538	0.9298	0.9403
Absolute Sum of Squares	0.4688	0.9825	0.7360

Figure 12. GBR12935 binding to *ex vivo* striatal synaptosomes prepared 4 weeks after mice were treated with PM. Left, isotherm binding plots; Right, kinetic and statistical parameters for the data.

c. Compare the extent of toxin-dependent actions on mitochondrial function in striatal synaptosomes by measuring thiazolyl blue dehydrogenase activity.

No experiments of this type were run in the past year.

d. Search for anatomical evidence of general neurotoxicity within light microscopic preparations of the nigro-striatal system by examining glial fibrillary acidic protein (GFAP) immunoreactivity as a marker for gliosis.

e. Search for anatomical evidence of general neurotoxocity within specific dopaminergic components of the nigro-striatal system using immunocytochemical staining for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH).

f. Confirm whether functional changes in dopamine transport are due to fluctuating levels of dopamine transporter (DAT) protein using immunocytochemical identification.

Methods: These biomarkers are dealt with together since fixation, sectioning, staining and analysis of the tissue is similar for all. In the previous reporting period (1/2000-1/2001), we presented data regarding the effects of individual administration of the insecticides PM and CPF upon striatal immunostaining for DAT and/or the dopamine synthesizing enzyme TH. These biomarkers were used to indicate, respectively, changes in the functional integrity of dopaminergic nigrostriatal terminals or degeneration of these terminals from the striatum. In the current reporting period, we have introduced the use of GFAP immunostaining as a more subtle indicator of nigrostriatal terminal insult. We have also examined the possibility of synergism between CPF and PM when administered together. In addition, we have increased the size of our sample for TH staining in mice receiving a high dose (200 mg/kg) of PM and we have refined our previous statistical analysis.

Insecticide-treated mice received either, 1) a high dose (200 mg/kg) of PM alone or 2) a high dose of PM in combination with 50 mg/kg of CPF. Each of these groups was paired with an equal number of matched vehicle-treated control mice. Dosing regimen was the same as that described in the original proposal. Sacrifice, perfusion, tissue staining, and quantitative image analysis of the tissue were performed by methods described in the original proposal and in the prior progress report. Alternate sets of tissue from each mouse were processed for immunostaining with polyclonal antisera to GFAP (6400:1) and TH (400:1) as well as for cresyl violet staining.

The statistical analysis used for our immunohistochemical data was modified from a non-parametric analysis to a more sensitive parametric analysis. This was done as follows. Scatterplots were used to assess the effects of microscope slide section position and date of tissue processing on differences in immunostained neuropil between paired sections. Based on this analysis, an ANOVA model, fitted using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) was used to correct for the effect of processing date on each data point. The corrected differences in immunostaining were then consistent with the assumptions for analysis by a paired t-test. For a pair of permethrin-treated and matched vehicle control brains, the difference in immunostained neuropil was averaged across microscope slides. Then, for each dose concentration group, the grand mean of these corrected mean differences was tested for its difference from zero using an alpha level of 0.05. Inflation of the type I error rate was avoided by treating each dose concentration group as a separate experiment. This new analysis did not change the outcome of our previously reported data (1/2000-1/2001). The statistical analysis of the data was conducted with the assistance of Dan Ward, statistical consultant for the College of Veterinary Medicine.

Results and discussion: Figure 13 is a box and whisker plot of the distribution of mean differences in TH and GFAP immunostaining, between matched pairs of PM-treated and vehicle control mice, where the dose of PM was 200 mg/kg (high dose). This dose of PM alone did not produce a significant change in the amount of TH immunopositive neuropil within the striatum compared to matched vehicle controls. The data shown for TH represents the pooling of 8 matched pairs of mice (each pair comprised of an insecticide-treated and matched vehicle control mouse) from the current reporting period with 8 matched pairs of mice from the previous reporting period. Neither set of TH data alone (not shown) revealed a significant change from zero and pooled data

were corrected for tissue processing date. Figure 13 also shows that although a high dose of PM did not change the amount of TH immunopositive neuropil compared to matched vehicle controls, it did produce a significant increase in GFAP immunopositive neuropil within the striatum (df = 15, p = .048).

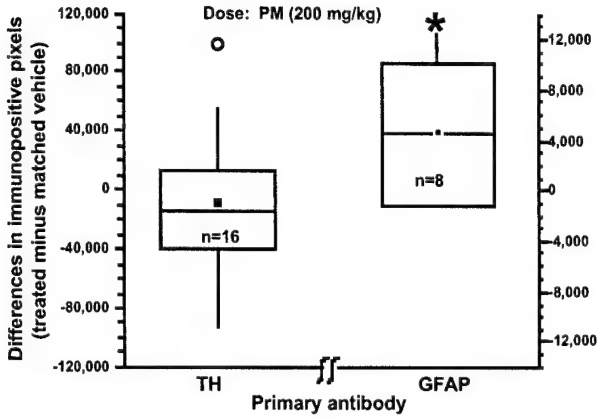


Fig. 13. Mean differences in TH and GFAP immunostaining, between matched pairs of PM-treated and vehicle control mice. Upper and lower tips of the whiskers, respectively, represent the minimum and maximum mean differences, while the height of the box represents the inter-quartile range. The line and black square within each box respectively represent the median and grand mean of the distribution of mean differences. The number (n) of matched pairs of treated and vehicle control mice is also indicated for each analysis. An open circle is used to represent a value that is more than 1.5 inter-quartile ranges above the median. A grand mean of zero represents no change in immunostaining between matched pairs of mice, while an asterisk indicates a significant non-zero difference between matched pairs.

Figure 14 is a box and whisker plot of the distribution of mean differences for TH and GFAP immunostaining, between matched pairs of vehicle control mice and mice treated with both 200 mg/kg of PM and 50 mg/kg of CPF. As can be seen in Figure 14, the combined insecticide treatment failed to produce a significant change in TH immunopositive neuropil within the striatum, compared with matched vehicle controls. However, this combined insecticide treatment produce a significant increase in striatal GFAP immunostaining (df = 7, p = .033).

These data reaffirm that degeneration of dopaminergic terminals in the striatum is an unlikely substrate for our previous finding that a high dose of PM decreases maximal dopamine uptake in this region. This conclusion is drawn from replicating the finding that a high dose of PM (200 mg/kg) fails to change the amount of striatal TH immunopositive neuropil compared with matched vehicle controls. However, the significant increase in GFAP immunoreactive neuropil in this region suggests that some form of tissue insult occurs in the striatum, following a high dose of PM, that is insufficient to induce degeneration of dopaminergic terminals within the temporal interval investigated. However, such damage may be sufficient to interfere with maximal dopamine uptake. Our previously reported immunohistochemical data (1/2000-1/2001), following a high dose of PM, suggest this damage is unlikely to be a change in the amount of DAT immunopositive neuropil.

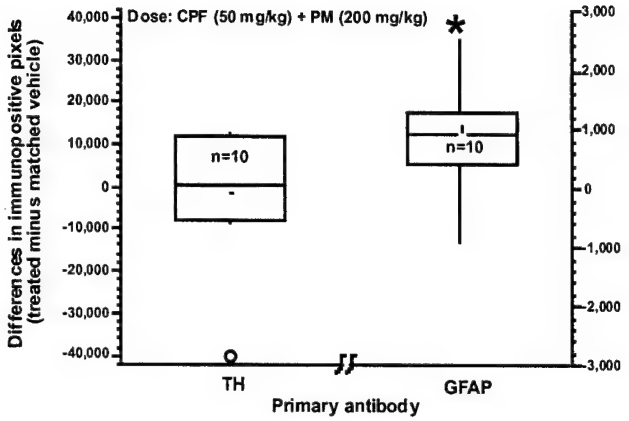


Figure 14. Box and whisker plot of the distribution of mean differences for TH and GFAP immunostaining, between matched pairs of vehicle control mice and mice treated with both 200 mg/kg of PM and 50 mg/kg of CPF. Figure displays statistical results as described above for Fig. 13.

We have previously reported (1/2000-1/2001) that a 50 mg/kg dose of CPF fails to alter the amount of TH immunopositive neuropil in the striatum, suggesting a lack of dopaminergic terminal degeneration. This dose is half of that which we have previously shown to alter open field behavior, dopamine turnover and muscarinic binding in the striatum and is therefore sub-threshold for significant alteration of striatal integrity. We have shown above that 200 mg/kg of PM also fails to alter the amount of TH immunopositive neuropil in the striatum, but that some form of tissue insult is indicated by an increase in GFAP immunoreactive neuropil. Our current finding that a combined dose of 200 mg/kg of PM and 50 mg/kg of CPF fails to produce a change in striatal TH immunopositive neuropil, suggests that a sub-threshold dose of CPF fails to act synergistically with PM to exacerbate the PM-induced tissue insult into full blown terminal degeneration. The tissue insult seen with the high dose of PM alone, as indicated by a significant increase in striatal GFAP immunostaining, persists in the combined treatment. However, the contribution of CPF to this effect is not clear since an analysis of GFAP has not yet been performed following a dose of 50 mg/kg of CPF alone. In the coming year, we intend to examine the possibility of synergistic effects of PM and CPF upon immunohistochemical changes in the striatum induced by MPTP.

g. Explore toxicant effects on open field/rearing frequencies and pole climbing behaviors and search for correlations between behavioral impairment and neurochemical effects.

Methods: Behavioral experiments focused on measurements of impairment of motor function. Alteration of motor behaviors was assessed using open field ambulation, rearing frequency, and a pole traction test similar to those described by Takahashi *et al.* (1989). Animals were tested at the end of the posttreatment period, prior to sacrifice for the neurochemical analyses. In the open field test, we assigned a movement unit whenever all 4 feet of the mouse entered a new square on the bottom of the arena. Movement units and the number of rearing behaviors produced were summed over a 3 min observation period. The number of rears was also summed for 3 min. The pole test apparatus consists of a 38 cm taped ring stand. Mice are placed at the top of the pole and allowed to hang by the forepaws. All mice are observed for up to 5 minutes. Times required for mice to turn (invert) and then climb down the pole are measured. Inversion times, climbing times, and the number of falls was determined.

Results and Discussion: Open field and rearing movements were unaffected by 10 mg/kg MPTP or any of the PM and CPF combination treatments (Fig. 15). Neither was there any significant effect on pole climbing behavior (data not shown). These results stand in contrast to

those using 30 mg/kg MPTP, where there was a definite trend towards a propensity to fall from the pole (Fig. 17, 2000 report).

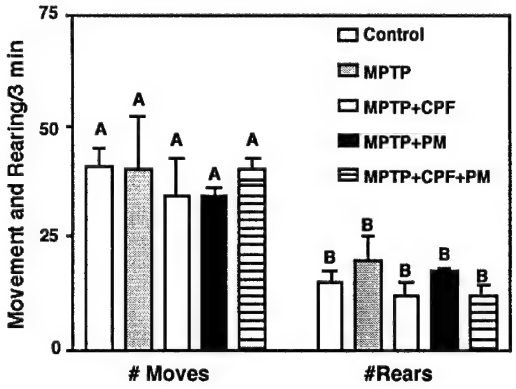


Figure 15. Movement and rearing following toxicant treatment. Abbreviations as defined previously and MPTP at 10 mg/kg, PM at 200 mg/kg, and CPF at 75 mg/kg. For movement or rearing measurements, bars labeled by different letters are significantly different (ANOVA with Student-Newman-Keuls post test, $p < 0.05$).

h. Determine the extent of acetylcholinesterase inhibition following treatment with toxicants for comparison with other behavioral and neurochemical effects.

No experiments on this biomarker were performed in the past year.

i. Define any toxicant-induced changes in cholinergic receptor density or function with respect to agonist-induced dopamine release from striatal synaptosomes.

Methods: This last objective of the research actually contains several separate neurochemical measurements. We have established methods for radioligand binding studies involving [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) and [^{3}H]epibatidine, with epibatidine binding adapted from the procedures of Houghtling *et al.* (1995). We have found that the very high affinity of this ligand is superior to that of labeled nicotine. In addition, two methods for measuring functional cholinergic modulation of dopamine release are still under development. The first will measure loss of label by repeated application of buffer with a pipettor. Alternatively, the labeled synaptosomes will have agonists superfused over them with a peristaltic pump and loss of label will be quantified in this manner. We have not yet performed any studies on the ability of cholinergic agonists to alter release of dopamine in striatal synaptosomes from insecticide-treated mice.

Results and Discussion:

In last year's report (Fig. 19, 2000 report), we found that exposing mice to PM caused an apparent upregulation of muscarinic receptors, as evidenced by an increase in the B_{max} for [^{3}H]QNB binding. Calculations of QNB B_{max} in the previous studies were faulty and resulted in erroneously high values for B_{max}. The error in our spreadsheets has been corrected and we obtain a B_{max} of 1-2 pmol/mg striatal protein in controls, which is in accord with other investigators (Schulte *et al.*, 1994). An upregulation of QNB binding by PM was confirmed (Fig. 16) and we expanded these results to a broader range of PM doses. Compared to controls, QNB binding was increased at 25 and 50 mg/kg PM, but was reduced at higher doses. QNB binding is also reduced at high doses of CPF, something we (2000 report, Fig. 20) and others (Chaudhuri *et al.*, 1993) have observed. Our initial results did not provide such a large downturn in QNB binding at high PM doses. Moreover, we have also observed a reduction in binding below baseline in at least one additional treatment group (Fig. 16). It therefore appears that muscarinic receptor density is rather precariously regulated in the presence of high doses of PM. At lower doses of PM (3-12 mg/kg) there was little dose-dependent change in QNB binding (data not shown). In previous studies, daily systemic injection of pyrethroids (*ca.* 1 mg/kg) in young mice slightly down-regulated cortical expression of muscarinic receptors (5-10%), but this effect was not observed in the striatum (Eriksson and Fredriksson, 1991).

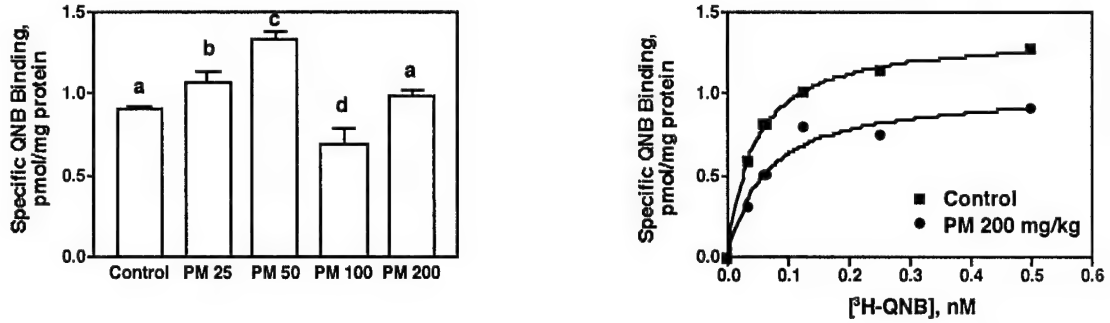


Figure 16. Enhancement of Bmax for QNB binding following PM treatment. Left: Bars represent means ± standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ($p < 0.05$). Bars labeled by different letters are significantly different. Right: Binding isotherm of control and PM-treated mice in another study.

Treatment	Specific binding, pmol/mg High affinity site (70 pM)	Specific binding, pmol/mg Low affinity site (2 nM)
Methoxytriglycol	0.097	0.263
PM, 200 mg/kg	0.040	0.136
Corn oil	0.015	0.058
CPF, 75 mg/kg	0.008	0.038

Table1. Specific binding of [^{3}H]epibatidine in striatal membranes from treated mice.

Previous studies have shown that the nicotinic receptor ligand epibatidine binds to high and low affinity sites having K_d values of in rat forebrain membranes of 16 and 391 pM, respectively, with about equal densities of both sites (Houghtling *et al.*, 1995). We have found similar K_d values of 6 and 225 pM in mouse synaptosomal membranes isolated from striatum. Based on these affinities, we have performed a screening study in striatal membranes isolated from mice treated with 75 mg/kg CPF and 200 mg/kd PM, compared to matched vehicle-treated controls (corn oil and methoxytriglycol, respectively). In order to estimate B_{max} values, single concentrations of ligand were run at 70 pM and 2 nM, with nonspecific binding defined by 300 μM nicotine (Houghtling *et al.*, 1995). The results of a preliminary study using these methods is shown in Table 1. Treatment with PM reduced high affinity binding site density 59% and low affinity binding site density 48%. Similarly, CPF reduced high affinity binding site density 47% and low affinity binding site density 34%. This latter result is similar to that of Zheng *et al.* (2000), who found that 14 daily oral doses of 7.5 mg/kg CPF reduced low affinity (1 nM) epibatidine binding sites 32% in adult rats. Thus, it would appear that neuroexcitatory toxicants down regulate presynaptic nicotinic receptors, although much further work needs to be done. We were surprised to see such large differences in binding in the two control groups. This difference merits further study.

Due to lack of available personnel, no experiments on cholinergic modulation of dopamine release were performed in year 3 of the project.

KEY RESEARCH ACCOMPLISHMENTS

Our most significant observation in the past year was the discovery of the slow time course of DAT induction. This effect suggests that the time course of alteration of other biomarkers may be slower than anticipated as well, and that effects could occur at lower doses than previously observed.

We replicated and expanded studies on dopamine transporter and QNB binding analysis and are confident of our results.

Additional work extended studies of presynaptic nicotinic receptor regulation by establishing methods for measurements of epibatidine binding.

Immunocytochemical studies revealed that GFAP is increased in insecticide-exposed mice.

REPORTABLE OUTCOMES

Meeting Presentations (speaker underlined)

J. R. Bloomquist, Pesticides and Parkinsonism: Military Insecticide Exposures and Its Relevance to the General Public. Spring 2001, Invited Speaker, Capital Chapter of the National Parkinson Foundation, Parkinson's Community Support Group, Fairfax, Virginia.

J. R. Bloomquist, Pesticides and Parkinsonism in Gulf War Syndrome. Spring 2001, Invited Speaker, Department of Toxicology, North Carolina State University, Raleigh, North Carolina.

J. R. Bloomquist, Insecticide Exposure, Dopamine Neurotoxicity, and Parkinson's Disease. Spring 2001, Invited Speaker, Department of Entomology, University of California, Davis, California.

J. R. Bloomquist, Insecticide Exposure, Dopamine Neurotoxicity, and Parkinson's Disease. Summer 2001, Invited Speaker, Department of Entomology, University of California, Riverside, California.

J. R. Bloomquist, Impact of Organochlorine, Pyrethroid, and Organophosphate Insecticides on Striatal Neurochemistry, Summer 2001, Nineteenth International Neurotoxicology Conference: Parkinson's Disease, Environment and Genes, Colorado Springs, Colorado.

Publications

D. Karen, W. Li, P. Harp, J. Gillette, and J. Bloomquist, Striatal Dopaminergic Pathways as a Target for the Insecticides Chlorpyrifos and Permethrin. *NeuroToxicology* (in press). A copy is included in the Appendix of this report.

J. Bloomquist, R. Barlow, J. Gillette, W. Li, and M. Kirby, Selective Effects of Insecticides on Nigrostriatal Dopaminergic Nerve Pathways. *NeuroToxicology* (in review, for the Proceedings of the 19th International Neurotoxicology Conference: Parkinson's Disease, Environment and Genes). A copy is included in the Appendix of this report.

Submission of a paper on cholinergic markers awaits the addition of synergism data on QNB binding. We previously anticipated submitting this paper in 2001. However, after scanning the published literature, we decided that additional information from combination studies with MPTP should be added to the manuscript. Acetylcholinesterase studies from synergistic treatments (Fig. 18, 2000 report) have been added to the paper, and the QNB binding studies should be done soon.

We also planned a 2001 paper on the reversibility of DAT induction, but given the surprising data on the time course of this effect, have held off on writing it up so that these new findings could be replicated and included in the manuscript.

CONCLUSIONS

The enhancement of the dopamine-depleting effect at 30 mg/kg MPTP observed previously with insecticides does not extent to lower doses of this Parkinsonian toxin.

Upregulation of the DAT is of greater amplitude and slower time course than previously observed, and through an interaction with a-synuclein, may provide a mechanism for pyrethroid-induced neurodegeneration.

Insecticide exposure down-regulates presynaptic nicotinic receptors.

GFAP expression seems to be a more sensitive indicator of toxic insult than TH levels.

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APPENDICES

Meeting Abstracts (formatted as per different society guidelines)

Not applicable for the talks presented.

Manuscripts and Papers:

Manuscript in Review

For: Proceedings of the Nineteenth International Neurotoxicology Conference: Parkinson's Disease, Environment and Genes, Colorado Springs, Colorado.

SELECTIVE EFFECTS OF INSECTICIDES ON NIGROSTRIATAL DOPAMINERGIC NERVE PATHWAYS

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Running Title: Dopamine Selectivity of Insecticide Action

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ABSTRACT

A degeneration of the nigrostriatal pathway underlies Parkinson's disease, and we have investigated the actions of insecticides on this pathway. For *in vivo* exposures, C57BL/6 mice were treated three times over a two week period with heptachlor, the pyrethroids deltamethrin and permethrin, or chlorpyrifos. Heptachlor and the pyrethroids increased maximal [^{3}H]dopamine uptake in striatal synaptosomes from treated mice, with dose-dependent changes in V_{max} displaying a bell-shaped curve. Western blot analysis confirmed increased levels of dopamine transporter protein in the striatum of mice treated with heptachlor and permethrin. In contrast, we observed a small, but statistically significant decrease in dopamine uptake by 100 mg/kg chlorpyrifos. For heptachlor, doses that upregulated dopamine transporter expression had little or no effect on serotonin transport. Permethrin did cause an upregulation of serotonin transport, but required a 30-fold greater dose than that effective on dopamine uptake. Other evidence of specificity was found in transmitter release assays, where heptachlor and deltamethrin released dopamine from striatal terminals with greater potency than other transmitter types. These findings confirm that insecticides possess specificity for effects on striatal dopaminergic neurotransmission.

Key words: Parkinson's disease, permethrin, deltamethrin, heptachlor, chlorpyrifos, dopamine transporter, dopamine release

FOOTNOTES

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INTRODUCTION

Parkinson's disease (PD) results mainly from a degeneration of dopaminergic fibers of the nigrostriatal pathway (Bowman and Rand, 1984). Among the possible causes of PD, there is a consistent epidemiological linkage with pesticide exposure (Semchuk *et al.*, 1992; Butterfield, *et al.*, 1993; Gorell *et al.*, 1998). Hazards from exposure to heavily used insecticides, such as the pyrethroids and organophosphates, exist from the manufacture, storage, spraying, and contact of personnel with insecticide-contaminated food or areas. Similarly, human exposure to organochlorine insecticides continues to be an important issue in human health, since the

environmental persistence of these compounds has raised concerns regarding their documented ability for bioaccumulation (Matsumura, 1985).

In addition, several studies observed effects of organochlorines consistent with parkinsonism. Early studies showed that rats fed 50 ppm dieldrin for 10 weeks had small reductions in whole brain dopamine after 4 weeks, but no change in the amount of striatal dopamine, even though striatal serotonin and norepinephrine levels were depressed (Wagner and Greene, 1974). Similarly, Mallard ducks (Sharma, 1973) and ring doves (Heinz *et al.*, 1980), fed dieldrin also displayed significant reductions in brain dopamine. Chemical analysis of Parkinsonian human brain found that it contained significantly greater levels of organochlorines, especially dieldrin, than healthy brain (Fleming *et al.*, 1994; Corrigan *et al.*, 1998). In studies with mesencephalic neuron cultures, dieldrin application caused cytotoxicity in dopaminergic neurons more so than GABAergic neurons, suggesting some selectivity of action (Sanchez-Ramos *et al.*, 1998).

We observed that the organochlorine insecticide heptachlor upregulated dopamine transport in striatal synaptosomes from treated mice at relatively low doses (Kirby and Bloomquist, 1996). Subsequent work expanded these findings, and showed that organochlorine and pyrethroid insecticides affect dopamine transporter (DAT) expression and dopamine release in the striatum (Kirby *et al.*, 1999; Kirby *et al.*, 2001; Karen *et al.*, 2001). The present paper will review our published work on these insecticides, and provide additional data confirming and extending our observations of the selective actions of insecticides on dopaminergic pathways.

MATERIALS AND METHODS

Chemicals.

Analytical grade chlorpyrifos (≥99%) was obtained from ChemService, Inc (West Chester, PA). Permethrin (a mixture of four *R,S-cis* and *R,S-trans* isomers) was obtained from Sigma Chemical Co. (St. Louis, MO), and deltamethrin (a single, 1*R-cis*, α-*S* isomer) were supplied by Crescent Chemical Co.(Hauppauge, NY). Analytical grade (≥99% purity) heptachlor was purchased from Chem Service (West Chester, PA). [^{3}H]Dopamine (20.3 Ci/mmol) and [^{3}H]GABA (100 Ci/mmol) were purchased from New England Nuclear (Wilmington, DE). [^{3}H]Glutamate (56.0 Ci/mmol) and [^{3}H]serotonin (17.9 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Buffer constituents were obtained from Sigma Chemical Co. or Fisher Scientific Co. (Pittsburgh, PA).

Animals and Treatments.

For experiments on insecticide-dependent changes in transporter regulation, we used male C57BL/6 retired breeder mice, purchased from Harlan Sprague-Dawley (Dublin, VA) that were 7-9 months old (28-40 g live weight). Treatments were administered three times over a two week period

according to the method of Kirby *et al.* (1999). All insecticides were delivered in methoxytriglycol (MTG) vehicle and injected ip, except CPF, which was carried in corn oil vehicle and given sc. Control mice received 50 µl corn oil or 10 µl of MTG vehicle alone. On the day following the last treatment, mice were killed by cervical dislocation and tissues were collected for neurochemical analysis.

Dopamine and Serotonin Uptake Studies.

Neurotransmitter uptake studies were performed according to the methods of Kirby *et al.* (1999) for dopamine and Kirby *et al.* (2001) for serotonin. Briefly, crude synaptosomes were prepared from fresh striatal (for dopamine) or cortical tissue (for serotonin) dissected from treated mice. Striatal or cortical dissections were performed using well-established landmarks evident in published figures of rodent brain (Cooper *et al.* 1986). Synaptosomes were incubated with various concentrations of [^{3}H]dopamine or [^{3}H]serotonin for 2 min. Uptake was stopped by addition of ice cold buffer, followed by vacuum filtration, washing, and liquid scintillation counting. Aliquots of each synaptosomal preparation were frozen at -20 °C for membrane protein determination, which was performed according to the method of Bradford (1976). Uptake rates were calculated by the method of Krueger (1990), using incubations with and without sodium ions (equimolar choline chloride substitution) to correct for nonspecific uptake. Uptake parameters (V_{max} and K_m) were determined by nonlinear regression to isotherm plots using Prism™, (GraphPad Software, San Diego, CA).

Western Blot Analysis.

Western blots were used to quantify the amount of DAT protein present in samples of brain tissue from treated mice. Crude synaptosomal membranes were prepared as previously described (Kirby *et al.*, 2001) and then homogenized, denatured, and the proteins separated by SDS-PAGE electrophoresis (Laemmli, 1970). Separation was accomplished on an 10% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane for one hour at 100V, according to the method of Towbin *et al.*, 1979). Blots were then incubated in 4% dry milk for one hour, followed by a one hour incubation in anti-DAT antibody (rat, Chemicon, Temecula, CA) and one hour with peroxidase-linked secondary antibodies. The blots were visualized using the ECL Chemiluminescense detection kit (Amersham, Buckinghamshire, England), with exposure to the ECL reagent for one minute, followed by autoradiography for various lengths of time, up to two minutes. Analysis of the blots was performed by digital image analysis using a Kodak Digital Camera and EDAS 290 System (Eastman Kodak Scientific Imaging Systems, Rochester, NY).

Neurotransmitter Release

Conventional neurotransmitter release assays in striatal, or cortical synaptosomes were performed essentially as described in Kirby *et al.* (1999). Crude synaptosomes were prepared from the two tissue sources and the final pellets resuspended in incubation buffer containing either

100 nM [^{3}H]dopamine, 115 nM [^{3}H]serotonin, 40 nM [^{3}H]GABA or 90 nM [^{3}H]glutamate (5 min, 37 °C). Cortical tissue was used as a synaptosome source for assays with [^{3}H]serotonin, due to the relatively low density of serotonergic terminals in the striatum. After loading, the membranes were then centrifuged and the labeled pellets resuspended in buffer and incubated with toxicants for 10 min at 37 °C. Lipophilic toxicants were dissolved in DMSO and final DMSO concentrations in incubations did not exceed 0.1%, with controls receiving 0.1% DMSO alone. Synaptosomes were diluted with 3 ml of wash buffer (37 °C), vacuum-filtered, and then washed 3 times with 3 ml of 37 °C wash buffer. Radioactivity on the filters was determined as described above.

Statistical Analysis.

For uptake studies, kinetic parameters (K_m and V_{max}) were determined by nonlinear regression to isotherm plots (GraphPad Software, San Diego, CA). For release studies, data were analyzed by nonlinear regression to a four parameter logistic equation using either Prism 2.0 (GraphPad Software, San Diego, CA) or MacCurveFit 1.3 (Kevin Raner Software, Victoria, Australia), which gave similar results. EC_{50} values for release of different neurotransmitters or dopamine uptake parameters following insecticide treatment were compared by T-test or by one-way ANOVA with Student-Newman-Keuls means separation test (InStat 2.03, GraphPad Software, San Diego, CA).

RESULTS

Dopamine uptake in striatal synaptosomes from treated mice was saturable and showed a good fit to a Michaelis-Menten model (r^2 values ≥ 0.98). Permethrin treatment did not have any significant effect on the apparent K_m of the dopamine transporter (range: 375-545 nM) in any of the treatment groups. Maximal dopamine uptake in C57BL/6 mice treated with permethrin varied with the dose (Fig. 1A), and peaked at a dose of 1.5 mg/kg. At this dose, dopamine uptake was significantly greater (34%) than that of the control value, while at higher doses maximal uptake declined. A similar dose-response profile was observed for heptachlor. Increases in V_{max} had a threshold dose of 3 mg/kg and uptake was maximally enhanced at 6 mg/kg (Fig. 1B). Maximal induction of V_{max} for striatal dopamine transport was 217% of control at this dose. Again, at higher doses, transport declined. Changes in apparent K_m did not match in a dose-dependent way the increases in V_{max} and displayed a variable response to heptachlor treatment (Kirby et al., 2001). In contrast, treatment of mice with chlorpyrifos caused a significant 10% reduction in dopamine uptake V_{max} at 100 mg/kg, the highest dose administered. No significant effect on V_{max} was observed at lower doses of chlorpyrifos.

Upregulation of the DAT was confirmed in striatal tissue taken from treated mice (Fig. 2). In these studies, there is clear evidence of greater antibody labeling, especially at the 0.8 mg/kg dose of permethrin. In the Western blot shown in Figure 2, it appears that two bands are labeled and both are apparently upregulated. In addition, we have observed that permethrin-dependent

upregulation of the DAT is still present four weeks after the last 0.8 mg/kg treatment (data not shown).

Levels of cortical serotonin uptake following heptachlor treatment did not reflect the increase in maximal uptake observed for dopamine (Fig. 3). These experiments used 6 mg/kg and 12 mg/kg heptachlor; the doses that in previous experiments produced the greatest increases in V_{max} for dopamine uptake. In these studies, there was no significant change in V_{max} from control (5.1 $\pm$0.1 pmol/min/mg) in mice treated with 6 mg/kg heptachlor (5.0 $\pm$ 0.2 pmol/min/mg). There was a statistically significant, 21% reduction in calculated maximal rates of serotonin uptake for 12 mg/kg heptachlor-treated mice (4.1 $\pm$ 0.3 pmol/min/mg). However, this reduction was essentially attributable to a more variable decrease in uptake at 1 µM serotonin only, since the other points on the curve (Fig. 3) overlapped closely those of the other treatment groups. No statistically significant change in K_m values was measured for serotonin uptake (range: 90-140 nM) for any of the heptachlor-treated mice.

Reduced sensitivity of cortical serotonergic pathways was also observed with permethrin treatment. *Ex vivo* cortical synaptosomes showed a dose-dependent upregulation of serotonin transport (Fig 4), but at doses at least 30-fold greater than that required to upregulate dopamine uptake. Upregulation was essentially complete at 100-200 mg/kg permethrin, and virtually disappeared as the dose was lowered to 25 mg/kg (Fig. 4). In addition, the maximal extent of upregulation of serotonin transport (34%), was identical to that observed for dopamine. There was no statistically significant effect on the K_m values for serotonin transport (range: 69-289 nM).

Differential sensitivity of nerve terminals to pyrethroid- or heptachlor-evoked release of neurotransmitter was observed in synaptosome preparations from striatum and cortex of ICR mice (Table 1). The pyrethroid deltamethrin released a variety of neurotransmitters from preloaded synaptosomes at nanomolar concentrations, with the EC_{50} for dopamine release being 2.4- and 8.6-fold more potent than serotonin or glutamate release, respectively. The EC_{50} for heptachlor-evoked release of [^{3}H]dopamine from striatal synaptosomes (low micromolar range) was about 23-fold less potent than that of deltamethrin. Compared to dopamine, the EC_{50}s for heptachlor-induced release of GABA and glutamate from striatal synaptosomes were significantly greater (*ca.* 7-fold and 13-fold, respectively). Serotonergic terminals were the least sensitive of the nerve terminal types tested for heptachlor-evoked release and were approximately 23-fold less sensitive than striatal dopaminergic terminals (Table 1).

An effect on dopamine release was also observed, *in vitro*, with chlorpyrifos (Fig. 5). Synaptosomes preloaded with dopamine were induced to release label at micromolar concentrations of chlorpyrifos, and the EC_{50} for this compound was calculated to be 3.8 $\pm$ 1.3 µM. The inhibition was also complete, and showed an excellent fit to a sigmoidal model (r^2 = 0.998). No experiments with chlorpyrifos were attempted using other neurotransmitters.

DISCUSSION

The ability of insecticide exposure to increase dopamine transport reflects a potent action, *in vivo*. We demonstrated this effect previously for the pyrethroid insecticide deltamethrin, which increased dopamine uptake by 70% following three doses of 6 mg/kg (Kirby *et al.*, 1999). Similarly, permethrin at 1.5 mg/kg increased significantly the maximal transport of dopamine, and this dose is about 3 orders of magnitude below the mouse ip LD_{50} for this compound (Gray and Soderlund, 1985). Moreover, technical permethrin is a mixture of four (1*R,S cis* and 1*R,S, trans*) isomers, only one of which (1*R*, *cis*) causes lethality in mammals (Casida *et al.*, 1983). If the 1*R*, *cis* isomer alone is responsible for the up-regulation, it comprises only 25% of the applied dose, and it was therefore present at about 0.4 mg/kg. A potent enhancement of uptake also occurs following treatment with heptachlor, where 6 mg/kg represents about 4% of the LD_{50} dose (145 mg/kg by ip injection; Cole and Casida, 1986). We assume that the observed increase in dopamine uptake was compensatory for increased levels of free synaptic dopamine, *in vivo*, and that balanced neurotransmission was maintained by increased expression of the transporter.

A greater abundance of DAT protein was confirmed in Western blots at doses of 0.8 and 1.5 mg/kg of permethrin. The apparent greater expression at 0.8 mg/kg, where transport was not upregulated, can be ascribed to different responses of different cohorts of mice, and the neurochemical effects observed vary from group to group with respect to dose. Previous work has shown that heptachlor-dependent increase in dopamine transport was also accompanied by an increase in DAT protein labeling in western blots of striatal membranes (Miller *et al.*, 1999). Because persistent effects on DAT expression occur at low doses, it appears to be a sensitive index of subclinical toxicant insult and should be investigated further as a biomarker of environmental toxicant exposure. In addition, a recent study by Lee *et al.* (2001) observed an interaction between the DAT and _-synuclein in cultured neurons. This interaction led to increased dopamine uptake and dopamine-induced cellular apoptosis, and would provide a mechanism whereby increased DAT expression by insecticides could play role in the development of PD.

For both permethrin and heptachlor, transport declined at doses greater than those causing a maximal induction of dopamine uptake. This decline most likely occurred from the inability of the synaptosomes to retain dopamine, rather than a down regulation of the DAT. For both compounds, there is evidence of cell stress occurring at higher doses. In mice treated with doses $\geq$12.5 mg/kg permethrin, less activity was present in a synaptosomal MTT dehydrogenase assay (Karen *et al.*, 2001), which is a measure of mitochondrial function (Carmichael *et al.*, 1987). Similarly, polarigraphic measurements of mitochondrial respiration were reduced in striatal synaptosomes from mice treated with $\geq$25 mg/kg heptachlor (Kirby *et al.*, 2001). In contrast to permethrin and heptachlor, striatal dopamine uptake was not up-regulated at any dose of chlorpyrifos tested. In fact, at the highest dose (100 mg/kg), dopamine transport V_{max} was slightly decreased. As was the

case for elevated doses of permethrin, treatment with 100 mg/kg chlorpyrifos significantly depressed MTT dehydrogenase activity in *ex vivo* synaptosomes, suggesting chemically-induced nerve terminal stress (Kirby *et al.*, 2001).

Whereas heptachlor increased dopamine uptake in the striatum, no change in maximal rates of serotonin uptake was detected in the cortex. There was an upregulation of serotonin transport following permethrin treatment, but only at doses at least 30-fold greater than that required to upregulate the DAT. The lack of comparable effect on serotonin uptake by insecticides suggests less disruption of cortical serotonergic pathways *in vivo*, since compounds that either block uptake (fluoxetine; Hrdina and Vu, 1993) or inhibit synthesis of serotonin (*p*-chlorphenylalanine; Rattray *et al.*, 1996) will affect the expression of serotonin transporter in rat cortex.

Specificity in neurotransmitter release assays is evident in the greater sensitivity of nigrostriatal dopaminergic nerve terminals to insecticide-evoked release than those of either glutamatergic or GABAergic projections to the striatum, or serotonergic terminals in the cortex. Pyrethroids and organochlorines may affect release through an action on sodium channels (Kirby *et al.*, 1999) and calcium ion flux/homeostasis (Yamaguchi *et al.*, 1979; 1980), respectively. The greater potency of insecticide-induced dopamine release, *in vitro*, is mirrored in the ability of permethrin or heptachlor treatment to up-regulate dopamine, but not serotonin transport, in *ex vivo* synaptosomes from C57 mice.

The mechanism of dopamine release by chlorpyrifos is not obviously related to its well described action as an anticholinesterase, and we did not test its efficacy against other transmitters. However, Dam *et al.* (1999) found that this compound released about 20% of labeled norepinephrine from whole rat brain synaptosomes at 50 μg/ml (142 μM). Species, tissue, or transmitter selectivity could contribute to this difference. The release of norepinephrine was not antagonized by blockers of muscarinic (atropine) or nicotinic receptors (mecamylamine), so the mechanism remains unknown. The lack of DAT upregulation by chlorpyrifos treatment suggests that the release effect of this compound was not sufficient to induce transporter expression at the doses tested, or perhaps was interfered with by other poisoning processes.

Selective effects on dopaminergic nigrostriatal pathways is also observed with the mitochondrial poison, rotenone (Ferrante, *et al.* 1997; Betarbet *et al.*, 2000), and have been ascribed to a constitutive metabolic deficiency of nigral neurons (Marey-Semper *et al.*, 1993). This characteristic might play some role in the greater effect of different insecticide classes having different modes of action on striatal dopamine release, although other differences in the structure or function of the release machinery for different transmitter types might also be involved.

A role for excessive release of dopamine as a neurotoxic mechanism in PD is supported by other experimental evidence. The toxic nature of elevated dopamine levels has been shown following *in vivo* injection (Filloux and Townsend, 1993) and through exposure to cultured

neurons, *in vitro* (Ziv *et al.*, 1994). Similarly, the compounds reserpine and tetrabenazine cause release of neurotransmitter from nerve terminals (Mahata *et al.*, 1996), deplete dopamine and other monoamine levels in brain (Bowman and Rand, 1984), and Parkinsonism is a common side effect of their use as drugs in humans (Montastruc *et al.*, 1994). Insecticides may manifest similar effects.

Although these studies have shown specific effects of insecticides on the nigrostriatal pathway, we have failed to demonstrate a reduction in dopamine titers following heptachlor, chlorpyrifos, or permethrin treatment (Kirby *et al.*, 2001; Karen *et al.*, 2001). Loss of dopamine is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both cellular levels of dopamine and cell loss in the nigrostriatal pathway. In previous studies, we measured the total amount of striatal dopamine by HPLC (Bloomquist *et al.*, 1999; Karen *et al.*, 2001). This technique may have missed incipient effects of insecticides on dopamine levels, because natural loss of dopaminergic neurons in aging is countered by an increase in dopamine synthesis by the remaining neurons (Tatton *et al.*, 1991). In our studies, the lack of significant effect on dopamine titers by insecticides does not support a rapidly developing Parkinsonism following short term exposures to these compounds. Longer term studies, especially those at environmentally relevant exposures and in combination with other susceptibility factors (*e.g.*, mitochondrial compromise), should be undertaken to reveal any effects of insecticides on striatal dopamine levels under these conditions.

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TABLES

Table 1. Potency of insecticides for releasing different neurotransmitters from synaptosomes.

Insecticide	Brain Region	Transmitter	[1]EC_{50} ± SEM
[2]Deltamethrin	Striatum	Dopamine	48[a] nM ± 25
	Cortex	Serotonin	117[b] nM ± 37
	Cortex	Glutamate	412[b] nM ± 76
Heptachlor	Striatum	Dopamine	1.1[a] μM ± 1.1
	Striatum	GABA	7.3[b] μM ± 1.2
	Striatum	Glutamate	13.7[c] μM ± 1.2
	Cortex	Serotonin	25.9[d] μM ± 1.3

[1]EC_{50} values labeled by different letters are significantly different from each other, as found by one-way ANOVA and Student-Neumann-Keuls means separation test ($p<0.05$).

[2]Data taken from Kirby *et al.* (1999).

FIGURES

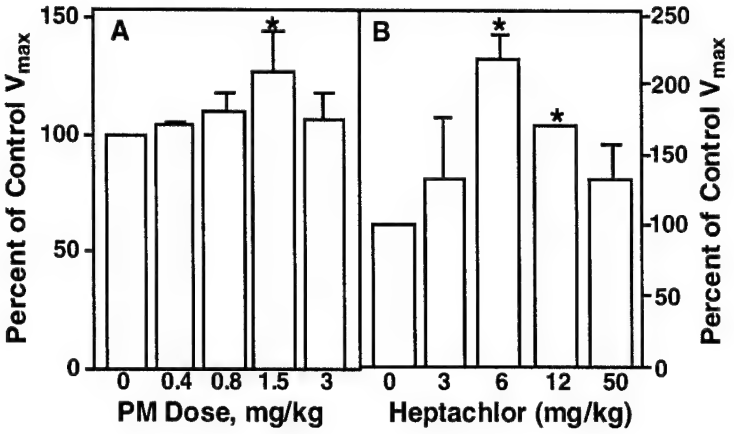

Figure 1. Representative bar graphs of dopamine transport after permethrin (A) or heptachlor treatment (B). Bars represent mean (with SEM) changes in V_{max} values, expressed as per cent of control and replicated across different cohorts of treated mice. Asterisks indicate effects significantly different from control using the untransformed data (T-test, $p<0.05$). The permethrin plot (A) is redrawn from Karen *et al.* (2001) and the heptachlor plot (B) is redrawn from Kirby *et al.* (2001).

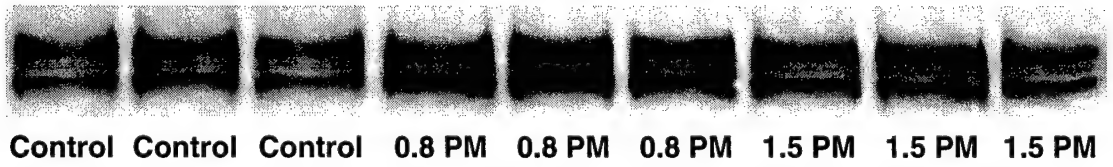

Figure 2. Western blot of striatal membranes taken from mice treated with the indicated doses of permethrin (PM) three times over a two week period. Each lane is from a pooled striatal homogenate, and run in triplicate.

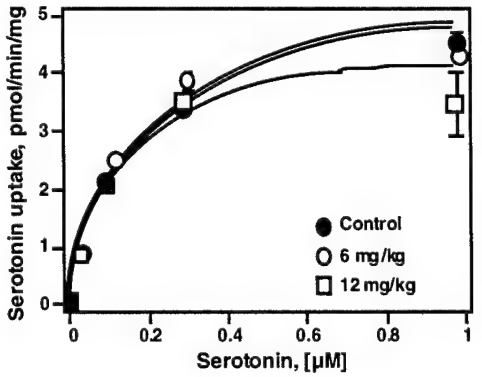

Figure 3. Serotonin uptake following treatment at doses of heptachlor known to upregulate dopamine transport. Symbols indicate means, along with the SEM. Lack of error bars indicates that the SEM was smaller than the size of the symbol. Redrawn from Kirby *et al.* (2001).

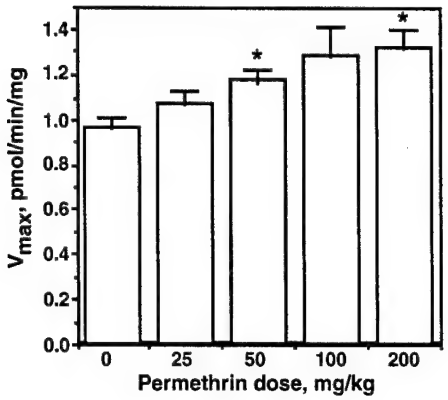

Figure 4. Serotonin uptake in cortical synaptosomes following treatment with permethrin. The results are taken from pooled membranes of single cohorts of mice having typically 5-6 animals. Asterisks indicate serotonin transport significantly different from control (T-test, $p<0.05$).

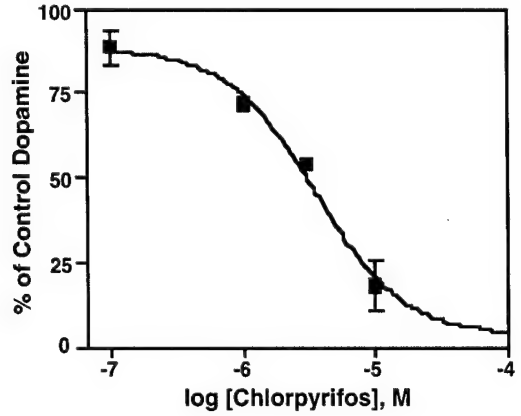

Figure 5. Ability of chlorpyrifos to cause dopamine release from striatal synaptosomes. Symbols represent means of three determinations with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.

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Striatal Dopaminergic Pathways as a Target for the Insecticides Permethrin and Chlorpyrifos

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Abstract

Because insecticide exposure has been linked to both Parkinsons disease and Gulf War illness, the neurotoxic actions of pyrethroid and organophosphate insecticides on behavior and striatal dopaminergic pathways were investigated in C57BL/6 mice treated with permethrin (three i.p. doses at 0.2–200 mg/kg) or chlorpyrifos (three s.c. doses at 25–100 mg/kg) over a 2-week period. Permethrin altered maximal [^{3}H]dopamine uptake in striatal synaptosomes from treated mice, with changes in V_{max} displaying a bell-shaped curve. Uptake was increased to 134% of control at a dose of 1.5 mg/kg. At higher doses of PM (25 mg/kg), dopamine uptake declined to a level significantly below that of control (50% of control at 200 mg/kg, $P < 0.01$). We also observed a small, but statistically significant decrease in [^{3}H]dopamine uptake by chlorpyrifos, when given at a dose of 100 mg/kg. There was no significant effect on the K_m for dopamine transport. Evidence of cell stress was observed in measures of mitochondrial function, which were reduced in mice given high-end doses of chlorpyrifos and permethrin. Although cytotoxicity was not reflected in decreased levels of striatal dopamine in either 200 mg/kg PM or 100 mg/kg CPF treatment groups, an increase in dopamine turnover at 100 mg/kg CPF was indicated by a significant increase in titers of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid. Both permethrin and chlorpyrifos caused a decrease in open field behavior at the highest doses tested. Although frank Parkinsonism was not observed, these findings confirm that dopaminergic neurotransmission is affected by exposure to pyrethroid and organophosphorus insecticides, and may contribute to the overall spectrum of neurotoxicity caused by these compounds. © *2001 Published by Elsevier Science Inc.*

Keywords: Parkinson's disease; Dopamine transport; Pyrethroid; Organophosphate; Gulf War illness

INTRODUCTION

The pyrethroid and organophosphorus (OP) insecticides are members of two chemical classes of heavily used compounds, and hazards from exposure to insecticides exist from the manufacture, storage, spraying, and contact of personnel with insecticide-contaminated food or areas. Among the possible hazards is a consistent epidemiological linkage between insecticide exposure and the incidence of Parkinson's disease (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998). Further, the neurological health problems which comprise Gulf War illness, reported by over 30,000 veterans, may be due to exposure of personnel to various chemicals. Specific compounds implicated include pyridostigmine bromide, the insecticides permethrin (PM) and chlorpyrifos (CPF), and the repellent *N,N*-diethyl-*m*-toluamide (DEET) (Abou-Donia et al., 1996; Abou-Donia et al., 2001). In previous studies, we have documented effects of the pyrethroid deltamethrin (Kirby et al., 1999) and the organochlorine heptachlor (Bloomquist et al., 1999; Kirby et al., 2001) on dopaminergic nerve pathways, which might be a contributory factor in the etiology of environmentally-induced Parkinson's disease (PD). The present study assessed effects on dopamine pathways following exposure to PM and CPF to ascertain whether damage to dopaminergic pathways and attendant Parkinsonism

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might be a consequence of Gulf War chemical exposures.

MATERIALS AND METHODS

Chemicals

Analytical grade CPF was obtained from ChemService Inc. (West Chester, PA). PM (a mixture of four *R,S-cis* and *R,S-trans* isomers) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co., St. Louis, MO, USA. [^{3}H]dopamine (20.3 Ci/mmol) was purchased from NEN Life Science Products Inc., Boston, MA, USA. Choline-Cl, KCl, $MgCl_2$, $CaCl_2$, and ascorbate were obtained from Sigma Chemical Co., Pargyline, D-glucose, sucrose, and HEPES were obtained from Fisher Scientific Co., Pittsburgh, PA, USA.

Animals and Treatments

Male C57BL/6 retired breeder mice were utilized for all experiments. Mice were purchased from Harlan Sprague–Dawley, Dublin, VA, USA and were aged 7–9 months (28–40 g live weight) at the time of the experiments. Mice were assigned randomly to treatment groups, which contained a minimum of six mice, so that the mean weight of all treatment groups was approximately equal. CPF carried in corn oil vehicle or PM carried in methoxytriglycol (MTG) vehicle were administered to the mice at multiple doses three times over a 2-week period according to the method of Bloomquist et al. (1999). CPF administration was by s.c. injection, while PM was administered by i.p. injection. Control mice received 50 μl corn oil or 10 μl of MTG vehicle alone. On the day following the last treatment day, mice were killed by cervical dislocation, and striatal tissues were collected at this time.

Dopamine Uptake Studies

Labeled dopamine uptake studies were performed according to the method outlined in Kirby et al. (1999). Briefly, crude synaptosomes were prepared from fresh striatal tissue dissected from treated mice, and incubated with [^{3}H]dopamine at various concentrations for 2 min. Transport of dopamine was determined after washing and vacuum filtration, followed by liquid scintillation counting. Uptake rates were determined by the method of Krueger (1990) in incubations with and without sodium ions (equimolar choline chloride substitution) in order to correct for low affinity transport. Uptake parameters (V_{max} and K_m) were determined by nonlinear regression to isotherm plots (Prism™, GraphPad Software, San Diego, CA, USA). Aliquots of each synaptosomal preparation were frozen at −20°C for membrane protein determinations, which was according to the method of Bradford (1976).

MTT Cytotoxicity Assay

This assay was run on synaptosomes by adapting the cultured cell methods of Carmichael et al. (1987) to synaptosomes. Striatal synaptosomes were prepared as described in Kirby et al. (1999) and incubated with MTT dissolved in Krebs–Henseleit buffer containing (mM): NaCl (140), KCl (5.0), $MgSO_4$ (1.3), $NaHCO_3$ (5.0), Na_2HPO_4 (1.0), HEPES (10), glucose (10), and $CaCl_2$ (1.2), pH 7.4. After 30 min at 37°C, the tubes were centrifuged for 5 min at $10,000 \times g$. The pellets were resuspended in DMSO to solubilize the formazan reduction product, and centrifuged again at $10,000 \times g$ for 1 min. Background absorbance of MTT (650 nm) was subtracted from test absorbance (580 nm) for the blue formazan product, both determined by a 96-well plate reader (Dynex Technologies Inc., Chantilly, VA, USA).

Dopamine and DOPAC Content

The methods employed were similar to those of Hall et al. (1992). Striata from individual mice were homogenized in 5% TCA containing 10 ng dihydroxybenzylamine (DHBA)/mg tissue wet weight as an internal standard and frozen at −70°C until analysis. Prior to analysis, samples were thawed and centrifuged at $10,000 \times g$ to pellet tissues. Dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were separated by HPLC using an ODS 3 μm Phase 2 column (3.2 mm × 100 mm). Mobile phase consisted of 170 mM NaH_2PO_4, 1.5 mM octanesulfonic acid, 5.5% methanol, 1.5% acetonitrile, and 93% H_2O, at a flow rate of 0.6 ml/min. Dopamine, DOPAC and DHBA standards were prepared to quantitate the amounts of dopamine and DOPAC in the samples.

Behavioral Assessments

On the last day of the study and prior to neurochemical analyses, behavioral effects were assessed by means of monitoring the number of open field movements and rearing frequency in an arena over a 3-min

period. The open field was the floor of a 10 gallon aquarium, divided into six equally sized squares. A movement was counted when the animal's front paws crossed any grid line. A rear was counted when the mouse raised onto its rear paws, lifting the front paws from the floor of the field.

Statistical Analysis

Statistical significance was determined using one-way ANOVA and Student–Newman–Keuls means separation if a statistically significant main effect of treatment was observed. Other statistical comparisons were by *t*-test calculations performed using InStat™ (GraphPad Software).

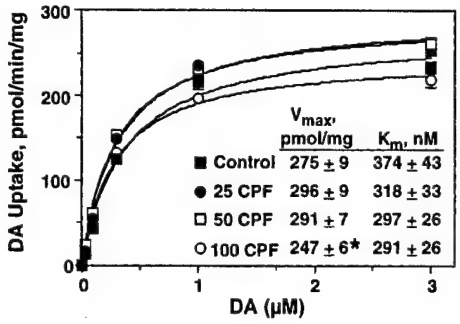

Fig. 2. Effect of CPF treatment on dopamine uptake in ex vivo striatal synaptosomes. Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol. Kinetic and statistical analysis is given in the inset table. Asterisks indicate an effect significantly different from control (*t*-test, $P < 0.05$).

RESULTS

Dopamine uptake rate in striatal synaptosomes displayed the expected saturation with increasing concentration of substrate (Fig. 1A). All curves showed a good fit to a Michaelis–Menten model, and typically had correlation coefficient (r^2) values ≥ 0.98. PM treatment did not have any significant effect on the apparent K_m of the dopamine transporter in any of the treatment groups. The control values for K_m averaged 233 ± 28 nm (mean ± S.E.M.). Maximal dopamine uptake in 9-month-old C57BL/6 mice treated with PM did vary with the dose, increasing at lower doses of PM, while at higher doses maximal uptake declined until it was less than the control level (Fig. 1A). Thus, a bar graph (Fig. 1B) of PM-induced V_{max} values from a broader range of doses took the form of a bell-shaped curve, in which the maximal rate of dopamine uptake, V_{max}, peaked at a dose of 1.5 mg/kg. At this dose, dopamine uptake was significantly greater (34%) than that of the control value, and this dose was replicated in four different groups of mice. All other doses were replicated at least twice. At higher doses of PM (>25 mg/kg), V_{max} declined to a level significantly below that of control (50% of control at 200 mg/kg) (Fig. 1B). Treatment of mice with CPF also caused a reduction in dopamine uptake V_{max} at the highest dose administered (100 mg/kg) (Fig. 2). Doses of CPF below 25 mg/kg were not tested in these experiments.

Striatal MTT dehydrogenase activity, an assay of mitochondrial integrity, was performed on pooled membranes from treated mice. Production of reduced formazan was reduced by 100 mg/kg, but not 50 mg/kg of CPF, which was actually higher than control (Fig. 3). The reduction caused by 100 mg/kg CPF was present at all concentrations of thiazolyl blue (MTT) tested, although only data at 0.55 mM is shown. PM also reduced MTT dehydrogenase activity, but at lower doses than CPF. At doses of 12.5, 25 and 50 mg/kg

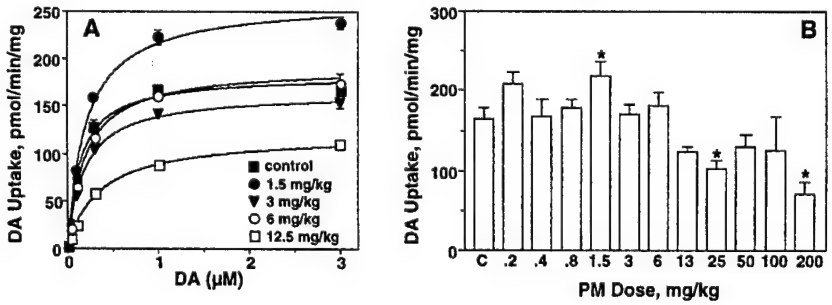

Fig. 1. Representative isotherm plots (A), and a bar graph (B) of the effects of PM on dopamine uptake. (A) Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol. (B) Percentage changes in maximal dopamine uptake (V_{max}) following PM treatment at the indicated doses. Asterisks indicate effects significantly different from control (*t*-test, $P < 0.05$).

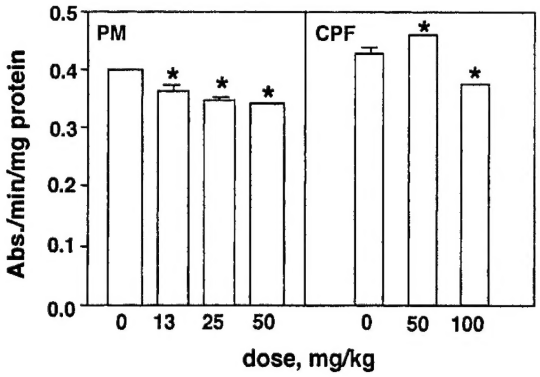

Fig. 3. Effect of PM (left) and CPF (right) treatment on mitochondrial activity (MTT reduction) in ex vivo striatal synaptosomes. MTT reduction activity is shown at a single concentration of 0.55 mM. In the bar graphs, the asterisk indicates an effect significantly different from control (ANOVA, $P < 0.05$).

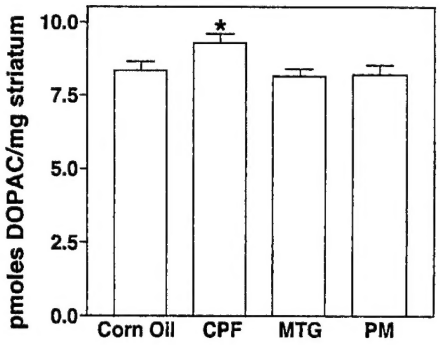

Fig. 4. Changes in DOPAC titers following treatment with vehicle (corn oil; MTG) or insecticide (100 mg/kg CPF; 200 mg/kg PM). Asterisks indicate effects significantly different from control (*t*-test, $P < 0.05$).

PM, MTT dehydrogenase activity was depressed 9, 12, and 14%, respectively. Similarly, there was a statistically significant 9% decrease in mitochondrial activity in a separate group of mice given 200 mg/kg PM.

CPF (100 mg/kg) increased striatal dopamine turnover, as indicated by significantly elevated titers of the dopamine metabolite, DOPAC (Fig. 4). The effect of CPF at this dose was an increase of 14% above control. In contrast, a high dose of PM (200 mg/kg) did not increase DOPAC titers. Neither CPF nor PM at these doses had any effect on striatal dopamine levels (data not shown).

CPF and PM both had similar dose-dependent effects on mouse behavior, according to movement and rearing tests. Statistically significant effects on rearing and movement were observed only at the highest doses of both compounds. Both movement and rearing frequency were decreased by treatment with 50 and 100 mg/kg CPF; however, the decrease at 50 mg/kg was not statistically significant (Fig. 5A and B). Similarly, high doses of PM decreased both frequency of open field movement and rearing frequency (Fig. 6A

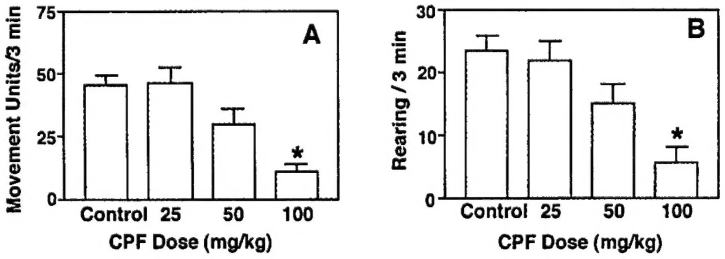

Fig. 5. Changes in open field (A), and rearing frequency (B) at the indicated doses of CPF. Asterisk indicates an effect significantly different from control (*t*-test, $P < 0.05$).

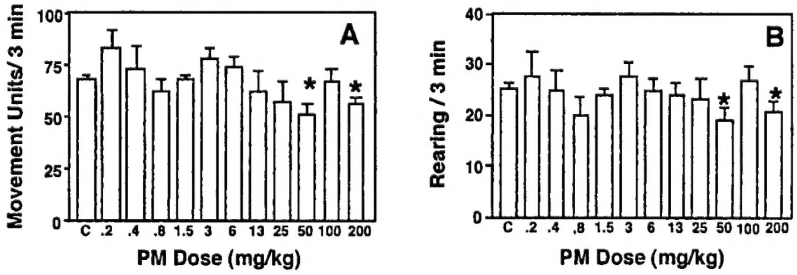

Fig. 6. Changes in open field (A), and rearing frequency (B) at the indicated doses of permethrin. Asterisk indicates an effect significantly different from control (*t*-test, $P < 0.05$).

and B). The effect was only significant at the 50 and 200 mg/kg doses, and not the 100 mg/kg dose, however.

DISCUSSION

The effect of 1.5 mg/kg PM for increasing the maximal transport of dopamine uptake is a potent action of this compound, in vivo. This dose is about three orders of magnitude below the rat oral LD_{50} for PM (Budavari et al., 1996), and we never observed any lethality at the highest dose (200 mg/kg) used in this study. Moreover, technical permethrin is a mixture of four (1*R*,*S*-*cis* and 1*R*,*S*-*trans*) isomers, only one of which (1*R*,-*cis*) has lethal effects in mammals (Casida et al., 1983). If the 1*R*,-*cis* isomer is responsible for the up-regulation, it is only 25% of the applied dose, and was actually given at about 0.4 mg/kg. We assume that the observed increase in dopamine uptake was compensatory for permethrin-dependent increases in dopamine release, in vivo. We have recently shown that the related pyrethroid deltamethrin releases a variety of neurotransmitters from preloaded synaptosomes, with the EC_{50} for dopamine release (48 nM) being 2.4- and 8.6-fold more potent than serotonin or glutamate release, respectively (Kirby et al., 1999). Thus, increased dopamine outflow was presumably balanced by increased uptake to maintain normal dopaminergic neurotransmission.

As the dose of PM increased, maximal transport of dopamine decreased to a level about 50% below that of controls, most likely from an inability of the synaptosomes to retain dopamine, rather than a true effect on dopamine transport. We would expect degeneration of the nerve terminals to be reflected in loss of striatal dopamine, which was not observed. However, there was evidence of cell stress in mice treated with doses $\geq$12.5 mg/kg PM in the MTT assay, which is a measure of mitochondrial function (Carmichael et al., 1987). In future studies, we expect to observe an up-regulation of dopamine transporter (DAT) protein in western blots at doses near 1.5 mg/kg of PM. Our previous work has shown that the organochlorine insecticide heptachlor increases dopamine transport in male C57BL/6 mice about two-fold at a dose of 6 mg/kg and this increase in uptake was accompanied by an increase in DAT protein labeling in western blots of striatal membranes (Miller et al., 1999). Moreover, the dose-response curve for heptachlor has a shape similar to that reported here for PM (Kirby et al., 2001). We have also demonstrated this effect for the pyrethroid insecticide deltamethrin, which increased dopamine uptake by 70% following three doses of 6 mg/kg (Kirby et al., 1999).

In contrast to PM, striatal dopamine uptake is not up-regulated by lower doses of CPF; however, in these experiments doses under 25 mg/kg CPF were not tested. At higher doses of CPF (100 mg/kg), dopamine transport V_{max} is significantly decreased, as was the case for high doses of PM. Similarly, at a dose of 100 mg/kg CPF, MTT dehydrogenase activity is significantly depressed compared to controls, suggesting chemically-induced nerve terminal stress.

CPF and PM failed to have an effect on striatal dopamine titers at the relatively high doses administered (data not shown). However, incipient effects on dopamine could be occurring that are masked when measured as total amount of striatal dopamine by HPLC. The effect may be similar to that seen in aged mice, in which 68% of the dopaminergic neurons are lost naturally, but there is a 103% increase in dopamine synthesis by the remaining neurons as a compensatory effect (Tatton et al., 1991). DOPAC levels were increased by treatment with a high dose of CPF, but not PM. Loss of dopamine and DOPAC is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both cellular levels of dopamine and cell death in the striatum. Elevated levels of DOPAC indicate greater turnover of dopamine in response to toxicant-induced processes (Hudson et al., 1985). We assumed that CPF increased turnover through neuronal hyperexcitation caused by inhibition of acetylcholinesterase, although interaction with other targets cannot be ruled out. The related compound methyl parathion, given at low doses (0.1 mg/kg per day for 15 days) to neonatal rats had little or no effect on dopamine content (Kumar and Desiraju, 1992). Soman induced an increase in DOPAC levels, consistent with an increase in dopamine turnover, but no change in dopamine levels (el-Etri et al., 1992; Fosbraey et al., 1990). We were somewhat surprised by the lack of any effect of PM on DOPAC, given that increased levels of striatal DOPAC had been demonstrated with this compound previously (Doherty et al., 1988). However the dose Doherty et al. used (1200 mg/kg, p.o.) probably gave a greater effective brain concentration than the treatment we used in this study (200 mg/kg, i.p.).

Movement, rearing and pole traction behaviors observed after CPF treatment are most likely due to inhibition of acetylcholinesterase activity, which is a hallmark of organophosphate exposure (Bowman and Rand, 1980). At doses above 25 mg/kg, there is a good correlation between dose and impairment of movement and rearing. PM has a less clear dose-dependent effect on behavior than CPF. However, at doses above 50 mg/kg, PM decreases both movement and rearing frequen-

cies. This action is consistent with results reported by Spinosa et al. (1999), in which movement and rearing frequencies were reduced by 10 and 30 mg/kg doses of the pyrethroid fenvalerate, which has greater mammalian toxicity than PM (Budavari et al., 1996). Although bradykinesia is a hallmark of PD (Bowman and Rand, 1980), it was not accompanied by a reduction in striatal dopamine in PM-treated mice, so another mechanism is probably responsible.

We have shown that up-regulated dopamine transport and mitochondrial integrity assays are sensitive biomarkers of exposure to certain insecticides. However, we do not know whether the neurochemical effects observed are persistent, or only temporary changes occurring after the last insecticide treatment. The lack of any effect on dopamine titers does not support a rapidly developing Parkinsonism following short-term exposures to these compounds. Long-term exposure studies should be undertaken. A recent study (Lee et al., 2001) observed an interaction between the DAT and α-synuclein in cultured neurons, which led to increased dopamine uptake and dopamine-induced cellular apoptosis. Such a scenario would provide a mechanism whereby increased DAT expression by insecticides could play role in the development of Parkinsonism.

Future studies will determine the extent of synergism in the effects of these insecticides on the nigrostriatal dopaminergic pathway, since synergistic neurotoxicity has been observed with DEET, pyridostigmine bromide, chlorpyrifos, and permethrin in various combinations (Abou-Donia et al., 1996; Abou-Donia et al., 2001). Other studies will address the extent of reversibility of the observed PM and CPF effects on striatal neurochemistry.

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